Tracing Evolutionary and Developmental Implications of Mitochondrial Stoichiometric Shifting in the Common Bean

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ABSTRACT

The recombination and copy number shifting activities of the plant mitochondrial genome are widely documented across plant genera, but these genome processes have not been as well examined with regard to their roles in plant evolution. Because of the extensive plant collections of *Phaseolus* spp and the degree to which cytoplasmic male sterility (cms) has been characterized in the common bean, this system would be valuable for investigating mitochondrial genome dynamics in natural populations. We have used the cms-associated sequence *pvs-orf239* as a mitochondrial genetic marker for these studies and have demonstrated its universal presence throughout a diversity of undomesticated Phaseolus lines. Within these populations, the *pvs-orf239* sequence is present in high copy number in ~10% of the lines, but substoichiometric in all others. This mitochondrial sequence, derived apparently by at least two recombination events, is well conserved with two point mutations identified that are both apparently silent with regard to the sterility phenotype. A putative progenitor sequence was identified in *Phaseolus glabelus* in substoichiometric levels, suggesting that the present-day *pvs-orf239* sequence was likely introduced substoichiometrically. Copy number shifting within the mitochondrial genome results in a 1000- to 2000-fold change, so that substoichiometric forms are estimated at less than one copy per every 100 cells. On the basis of PCR analysis of root tips, we postulate that a mitochondrial "transmitted form" resides within the meristem to assure transmission of a complete genetic complement to progeny.

THE mitochondrial genome of higher plants is unusual in its structure, distinguished by high and low frequency DNA recombination occurring between repeated sequences interspersed throughout the genome. Recombination activity at directly oriented repeats allows the subdivision of the genome into a complex, multipartite, highly redundant organization (Fauron *et al.* 1991). Although the regulation of this recombination activity is not yet understood, the complex and highly variable structure of the mitochondrial genomes of several plant species is well documented (Wolstenholme and Fauron 1995).

More recently, evidence has accumulated to suggest that an intriguing consequence of a multipartite genome configuration is the somatic modulation of mitochondrial genotype. A process of genomic shifting is prevalent in plants; this results in dramatic suppression in relative copy number of various low frequency recombination-derived subgenomic molecules within the genome. Stoichiometric shifting of the mitochondrial genome was first reported by Small *et al.* (1987) in studies of the maize *atpA* gene. At that time it was postulated that these striking copy number changes might represent a means of retaining genetic variation (Small *et al.* 1989).

Comparable copy number shifts within the mitochondrial genome have been observed in several plant species (Bonhomme *et al.* 1992; Vitart *et al.* 1992; Pla *et al.* 1995; Yesodi *et al.* 1995; Suzuki *et al.* 1996; Gutierres *et al.* 1997; Laser *et al.* 1997), with frequency influenced by growth conditions (Kanazawa *et al.* 1994) and nuclear genotype (Mackenzie *et al.* 1988). In the common bean (*Phaseolus vulgaris* L.), genomic shifting may have influenced the evolution of the species. A cytoplasmic male sterility-associated sequence, designated *pvs-orf239* (Chase and Ortega 1992; Johns *et al.* 1992), has been identified within the mitochondrial genome of particular lines of *P. vulgaris*, *P. coccineus*, and *P. polyanthus* (Hervieu *et al.* 1993). In *P. vulgaris*, introduction of the nuclear fertility restorer gene, designated *Fr*, by crossing produces a permanent, nonsegregating condition of male fertility in association with shifting of the *pvs-orf239* sequence to substoichiometric levels (Mackenzie and Bassett 1987; Mackenzie and Chase 1990; Janska *et al.* 1998). An apparently identical genomic shifting phenomenon is observed upon spontaneous reversion to

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TABLE 1

Genetic materials used in this study

Line	Nuclear genotype	*pvs-orf239*[a]	Phenotype
CMS-Sprite	*frfrfr2fr2*	HCN	Sterile
Sprite	*frfrfr2fr2*	LCN	Fertile
G-08063	*frfrFr2Fr2*	HCN	Fertile
R-351	*FrFrfr2fr2*	LCN	Fertile
G-11115	Unknown restorer	HCN	Fertile
WPR-3	*frfrfr2fr2*	LCN	Fertile
83-1	*frfrfr2fr2*	LCN	Fertile

[a] *Pvs-orf239* HCN and LCN designations refer to presence in high *vs.* low copy number, respectively.

fertility (Janska and Mackenzie 1993; Janska *et al.* 1998), with frequencies likewise dependent on nuclear background (Mackenzie *et al.* 1988).

The phenomenon of mitochondrial genomic shifting is apparently unique to higher plants and, although widespread, is not well understood. The magnitude of the copy number shifts to and from substoichiometric levels has not been determined, though estimates have been made (Laser *et al.* 1997), and the mechanism by which the genomic change occurs has not been defined. We have previously suggested that DNA replication is likely involved on the basis of observations with *in organello* incubations (Janska *et al.* 1998), and a developmental stage important to the process appears to be gametogenesis (He *et al.* 1995). The extent to which genomic shifting might occur within natural populations, and whether this mode of genotype modulation might play a role in species evolution, is unknown. Moreover, given the rapid and dramatic nature of the phenomenon, one must wonder how a full mitochondrial genetic complement is retained without the subsequent irreversible loss of genetic information that is copy number suppressed. Within this study we have addressed these questions in the common bean system. This particular genetic model was selected for these investigations by virtue of a vast collection of diverse, undomesticated plant materials available, the utility of a unique mitochondrial marker in the male sterility-associated *pvs-orf239* sequence, and the extensive genetic characterization of the cytoplasmic male sterility trait in common bean.

MATERIALS AND METHODS

Plant materials: To assess the frequency of *pvs-orf239* in *P. vulgaris* populations, a representative wild bean core collection of 105 accessions (Tohme *et al.* 1996) was used. In addition, 5 more wild accessions, 36 cultivated accessions, and 9 accessions related to the lima bean (*P. lunatus*) and related species from the Andean region were included. The *pvs-orf239* homologs from species *P. lunatus* (accession G26347) and *P. glabelus* (accession S29161) were derived for DNA sequence characterization. The seed, as well as some DNA samples, was kindly provided by the Centro Internacional de Agricultura Tropical (CIAT) at Colombia. A complete list of the accessions and their origins is provided in Appendix Table 1 at http://psiweb.unl.edu/appendix.html.

Genetic stocks and populations: The genetic resources utilized in this study are listed in Table 1. G-08063 is a fertile accession line that contains the fertility restorer gene designated *Fr2* (Mackenzie 1991). The G-08063 line also contains the same sterility-inducing mitochondrial genome configuration as the line designated CMS-Sprite (*frfrfr2fr2*; Mackenzie 1991), a male-sterile line derived by crossing the G08063 cytoplasm to a "Sprite" nuclear background (BC_{16}; Bassett and Shuh 1982). Sprite is a fully fertile snap bean cultivar that is isonuclear to CMS-Sprite but contains a normal fertile cytoplasm (the sterility-inducing mitochondrial sequence *pvs-orf239* is substoichiometric). Restorer line R-351 is a fertile line that contains the fertility restorer gene *Fr (FrFrfr2fr2*), is near-isogenic to CMS-Sprite, and contains *pvs-orf239* substoichiometrically. The genetic populations used in this study are listed in Appendix Table 2 at the web site http://psiweb.unl.edu/table2.html. The F_2 and F_3 generations were the product of self-pollination of the previous generation.

The male-sterile line CMS-Sprite undergoes spontaneous reversion to fertility at low frequency, accompanied by the genomic shifting of *pvs-orf239* to substoichiometric levels (Mackenzie *et al.* 1988; Janska *et al.* 1998). The independent, spontaneous revertants used in this study are designated WPR-3 and 83-1.

To characterize the effect of the nuclear restorer gene *Fr* on different mitochondrial genomic configurations, *P. vulgaris* accessions that contain the *pvs-orf239* sequence in different genomic environments were used for genetic crosses. These lines, G-11115, G-24711, and CMS-Sprite, were used as female parents in crosses by F_3 plants derived from [CMS-Sprite/R-351] BC_3 (*FrFr*) used as a source of the *Fr* gene. R-351 designates the original line from which *Fr* was first identified (Mackenzie and Bassett 1987). The genetic populations are listed in Table 2A and the crossing strategy for *Fr* introduction is indicated in Table 2B.

Plants were grown in the greenhouse with semicontrolled conditions. Generally, the temperature was 25° daytime and 18° nighttime. The daylight duration was 16 hr. All populations were developed using standard breeding methods. Fertility classification was based on three main criteria: pollen stainability (IKI stain), pollen shed, and seed set as described in detail previously (Mackenzie and Bassett 1987). Fertile plants produce >90% viable pollen and produce normal seed-bearing pods with no evidence of parthenocarpy. Sterile plants produce microspores aborted in tetrads, no visible pollen shed, and parthenocarpic pods bearing no seeds. Semisterile plants produce both fertile pollen and aberrant pollen in a single bud, giving rise to both seed-bearing and parthenocarpic pods on an individual plant.

DNA extraction: Genomic DNA was extracted from leaf tissue using the procedure of Vallejos *et al.* (1992). Cosmid and plasmid DNAs were prepared as mini-preps with the method described by Sambrook *et al.* (1989).

Root tip sections: Seeds of WPR-3 and CMS-Sprite lines were imbibed on two layers of water-soaked filter papers at room temperature for 3–5 days. Primary roots of ~2 cm in length were used. Two sections within the meristematic zone (~250 μm in length per section) were cut under a stereoscopic zoom microscope, Nikon model SM 2800. The root meristematic zone was considered for the purposes of this study as the zone within the root tip in which the cells are small, densely organized, with large nuclei (rapidly dividing) before the elongation zone. The meristem was, therefore, roughly calculated to extend to ~500 μm from the root tip including the root cap.

TABLE 2

A. Populations used to investigate *Fr* action on different mitochondrial types

Genetic cross	Generations
[G-11115/Sprite]BC_1//R-351[a]	F_2 and F_3
[G-24711/Sprite]BC_1//R-351	F_2 and F_3
CMS-Sprite/R-351	F_2 and F_3
WPR-3/G08063	F_1, BC_1, and BC_2

B. Genetic strategy to introduce *Fr* into two Phaseolus lines

Cross	*Pvs-orf239*	Nuclear background
G-11115/Sprite F_1[b]	+	*FrjfrjfrfrFrjfrjfrfr*[c]
G-11115/Sprite BC_1	+	*FrjfrjfrfrFrjfrjfrfr; frjfrjfrfr*
[G-11115/Sprite]BC_1//R-351[d]		
F_1 (sterile plants only)	+	*FrjfrjFrfr*
[G-11115/Sprite]BC_1//R-351 F_2	+	*frjfrjFrFr: frjfrjFrfr: frjfrjfrfr*
[G-11115/Sprite]BC_1//R-351 F_3		
(fertile plants only)	+	*FrjfrjFrFr*

[a] R-351 refers to [CMS-Sprite/R-351]BC_3F_3.
[b] Same genetic crosses were performed using G-24711 and CMS-Sprite (positive control).
[c] The nuclear restorer gene present in the different accessions is designated here *Frj*.
[d] R-351 line refers here to the BC_3F_3 line used as source of *Fr* gene.

Total genomic DNA from root sections was prepared with a DNA extraction technique for microscale specimens developed by Suzuki *et al.* (1995). Approximately 100 root sections were used for each DNA preparation.

PCR-based analysis in *P. glabelus*: To investigate the *pvs-orf239* homolog in *P. glabelus*, the longest *pvs-orf239*-related PCR product was amplified with the primers *orf239-glabF* and *orf239-glabR* (Table 3). These reactions were carried out in a total volume of 50 μl containing 100 ng of total genomic DNA, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 200 nM of each primer, 2 units of DyNAzyme II DNA polymerase (Finnzymes), and the buffer supplied by the manufacturer. PCR was performed in a UNO II thermal cycler (Biometra, Tampa, FL). The amplification was initiated with 3 min denaturation at 94°, followed by 30 cycles of 94° for 10 sec, 55° for 30 sec, and 72° for 1 min.

TABLE 3

Nucleotide sequences and corresponding designations for primers used in this study

Primer designation	Primer sequence (5′-3′)
orf239-5′	ACATGTTCCTCCCATTCA
orf239-3′	CCATTAGCGGGGATGCTT
cob-5′	ATGACTATAAGGAACCAA
cob-3′	TGGAATTCCTCTTCCAAC
cmsorf-5′	AGGGAGGATCCGTCCGAG
mutg-3′	CCAGGGCTTTTAAAACCCGAAT
orf239-A5′	ACATGTTCCTCCCATTCAACC
orf239-A3′	AAGATAGACGTCGCTCTCTGC
cox-5′	CAATAAAGTGAGGGCTTTCCG
cox-3′	GGGAATGCTGTTACTGGAACG
orf239-F	TTCCGCGTTCTCTTAAGTCG
orf239-R	GGAATCCATTTCTTCCACCA
cob-F	CCGCAATAGCACCAGTTTTT
cob-R	GTTGACATCCGATCCAACCT
orf239-glabF	AGATCCATACCGATTGAG
orf239-glabR	AGGCGTTGTTGTGCGATATCATC
F7	GACCATCTCAATCCGCAGGT
R7	CTAGGGGCATAGCCCTTCAC

Real time quantitative PCR: Real time quantitative PCR was performed using the primers *orf239-F*, *orf239-R*, *cob-F*, and *cob-R* (Table 3). The primers *orf239-F* and *orf239-R* amplify a 150-bp fragment internal to the *pvs-orf239* sequence. The primers *cob-F* and *cob-R* amplify a 171-bp fragment internal to the cytochrome b (*cob*) sequence. For α-amylase amplification, primers *F7* and *R7* were used to amplify a 100-bp fragment internal to the α-amylase gene from *P. vulgaris*.

Primer sequences used to amplify a portion of the α-amylase gene from *P. vulgaris* were designed from database accessions AB15131 for the *P. vulgaris* α-amylase mRNA sequence and accession X73301 for the genomic sequence of α-amylase in *Vigna mungo*. Amplification efficiency of primers for α-amylase was estimated at one-half that of primers for *pvs-orf239*. This estimate was based on quantification of agarose gel fractionated PCR products derived from serial dilutions of known template concentrations. The quantitations were conducted with the gel documentation 2000 system (Bio-Rad, Richmond, CA) and the Quantity One quantitation software.

Reaction mixtures (50 μl) consisted of 25 μl of SYBR Green PCR master mix (PE Biosystems, Foster City, CA) containing SYBR Green I dye, Amplitaq Gold DNA polymerase, dNTPs with dUTP, and optimized buffer components. Cycling conditions consisted of an initial denaturation step of 95° for 10 min as a "hot start," 40 cycles of 92° for 30 sec, 58° for 1 min, and 72° for 1 min and 30 sec, with a final elongation step of 72° for 5 min. The PCR reactions and quantifications were carried out in the real time PCR detection system iCycler iQ (Bio-Rad).

During PCR amplification fluorescence emission was monitored. The increase in fluorescence emission of the dye used is proportional to the amount of PCR product accumulated that, in turn, is proportional to the starting amount. A standard curve was constructed using templates of known copy number

TABLE 4

Distribution of the *pvs-orf239* sequence within genotypes of wild and cultivated *P. vulgaris*, *P. lunatus*, and related species

		Pvs-orf239 sequence		
Groups of accessions	No. assayed	HCN	LCN	Percentage HCN
Core collection wild/weedy	101	13	88	12.9
Wild/weedy	5	2	3	40.0
Cultivated	36	6	30	16.7
P. lunatus and related species	9	0	9	0.0
Total	151	21	130	13.9

HCN, high copy number; LCN, low copy number.

for the target sequence. The copy number of the samples was estimated by plotting the threshold cycle (the cycle at which fluorescence is considered to be significant above the background level and within linear range) against the log of the starting copy number (iCycler iQ real time detection system from Bio-Rad manual). To construct the standard curve, serial dilutions of cloned *pvs-orf239*, *cob*, and α-amylase fragments in PCRII Topo vector (Invitrogen, Carlsbad, CA) were used. The number of copies in each dilution was calculated with the following formula: (number of moles) (6.02×10^{23}) = number of copies. All standards and experimental samples were assayed in duplicate wells in one (revertant 83-1 sample), two (α-amylase), and four (CMS-Sprite and revertant WPR-3) independent trials.

Mitochondrial DNA probes and DNA cloning: Cosmid clones used in comparative DNA gel blot hybridizations were derived from mitochondrial DNA libraries of CMS-Sprite (Mackenzie and Chase 1990) and WPR-3 (Janska and Mackenzie 1993). The *pvs-orf239* probe was derived by polymerase chain reaction amplification using total genomic DNA as template.

Primers *orf239-5′* and *orf239-3′* were used to PCR amplify the *pvs-orf239* sequence from eight accessions of common bean. The derived 720-bp fragments were ligated to the PCRII vector (Invitrogen). DNA sequencing was conducted on the ALFexpress automated DNA sequencer system from Pharmacia Biotechnology. Sequence analysis was conducted using the GCG software with the BestFit program.

DNA gel blotting and hybridization procedures: Total genomic DNA samples were digested with restriction enzymes *Pst*I and *Eco*RI according to manufacturer's instructions (Promega, Madison, WI). Agarose gel electrophoresis, DNA transfer to Hybond-N nylon membrane (0.45 μm, Amersham, Arlington Heights, IL), extraction of DNA fragments, labeling of DNA, and filter hybridizations were conducted according to procedures described previously by Janska and Mackenzie (1993). Autoradiography was carried out by exposure to X-ray film (Fuji) with intensifying screens (Pickett) at −70°. Short exposure times were generally overnight and long exposure times were ~1 week.

RESULTS

***Pvs-orf239* exists universally as a conserved and functional sequence within undomesticated *P. vulgaris* populations:** The *pvs-orf239* mitochondrial sequence was first identified as a male sterility-inducing sequence in a highly selected breeding line designated G08063 (Mackenzie 1991; Chase and Ortega 1992; Johns *et al.* 1992), deriving from a cross between lines POP and NEP2 (CIAT Germplasm Collection Center, Cali, Colombia). A subsequent study showed that other Phaseolus species might contain the same or a related sequence (Hervieu *et al.* 1993). Consequently, we were interested to determine the extent to which *pvs-orf239* might be represented and conserved within undomesticated populations of Phaseolus. We surveyed a core collection of 105 wild and 36 cultivated accessions of Phaseolus from the different gene pools. This core collection, maintained at the CIAT, was designed to represent the genetic diversity that has been identified to date within the germplasm collection representing wild *P. vulgaris* and closely related species. The 36 cultivated lines were selected to represent most of the gene pools identified in beans.

Table 4 shows the results of our survey utilizing a PCR/DNA gel blot assay that allows the discrimination between high copy number and substoichiometric forms of *pvs-orf239*. Figure 1 shows an example of this assay. The detection of substoichiometric forms of *pvs-orf239* sequence was template concentration dependent. At high concentrations of template DNA, the PCR product could be visualized by ethidium bromide staining in agarose gels (data not shown), although it never approached the level of amplification achieved in accessions with *pvs-orf239* in high copy number. Accessions with *pvs-orf239* sequence in high *vs.* low copy number were also distinguished by hybridization of total genomic DNA using the PCR-amplified sequence as probe. High-copy-number *pvs-orf239* hybridization was visible by autoradiography after short exposure times (generally 5–16 hr). In the low-copy-number *pvs-orf239* lines no hybridization was detected unless films were exposed for extended periods (generally ranging from 3 days to 2 weeks depending on sample preparation).

We detected the presence of *pvs-orf239* in 100% of the lines that we surveyed, with the sequence substoichiometric in over 86% of the lines. Lines containing the sequence in high copy number are listed in Appendix Table 5 at Web site http://psiweb.unl.edu/table5.html. This observation, together with that of Hervieu

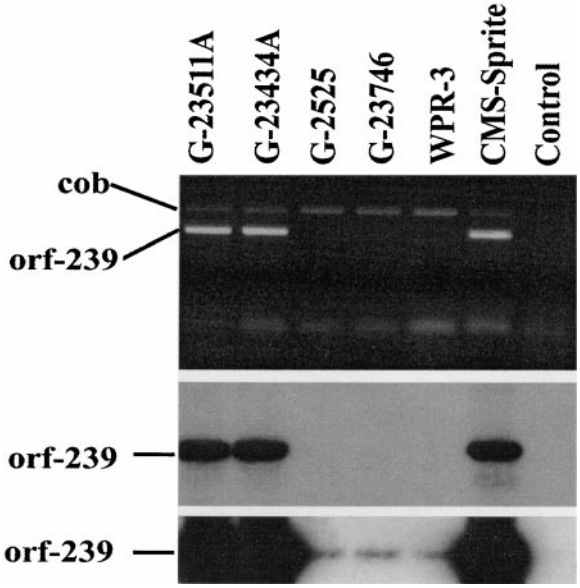

FIGURE 1.—Sample results from the survey for *pvs-orf239* demonstrating the contrast in relative copy number of the sequence. (Top) PCR amplification of *cob* (internal control for PCR reaction) and *pvs-orf239* sequences from different selected common bean accessions containing *pvs-orf239* in high (G-23511A, G-23434A, and CMS-Sprite) and low (G-2525, G-23746, and WPR-3) copy number. Samples were subjected to gel electrophoresis in 1% agarose. (Middle and bottom) DNA gel blot analysis of PCR products probed with ^{32}P-labeled *pvs-orf239* for exposure times of 2 hr (middle) and 3 days (bottom). Primers *orf239-5′*, *orf239-3′*, *cob-5′*, and *cob-3′* were used for the PCR reactions. The primer sequences are listed in Table 3. Primers *orf239-5′* and *orf239-3′* amplify a 720-bp fragment that encompasses the *pvs-orf239* sequence. Primers *orf239-5′* and *cob-3′* amplify a 783-bp fragment that contains the *pvs-orf239* sequence and the flanking 83 bp that share 100% homology with the 3′ end of apocytochrome b (*cob*). Primers *cob-5′* and *cob-3′* amplify a 1148-bp fragment that corresponds to most of the coding region of the *cob* gene. This amplified fragment represents an internal control for the PCR reaction. A 20-ng sample of total genomic DNA was used as template for the PCR reaction. PCR cycling conditions involved an initial denaturation step of 94° for 1 min, 35 cycles of 92° for 30 sec, 58° for 1 min, and 72° for 1 min and 30 sec, with a final elongation step of 72° for 5 min. Total genomic DNA samples prepared from CMS-Sprite and WPR-3 lines were used as controls for high *vs.* low copy number of *pvs-orf239*, respectively.

et al. (1993) showing that *pvs-orf239* or a related sequence is present in other Phaseolus species, suggests that the sequence arose prior to the divergence of the *P. vulgaris* species. To assess the extent to which the sequence has been conserved, we cloned eight alleles of the sequence, selected from diverse lines. From these, two variant sequences were observed, one detected twice in the sampling. Results of the sequence analysis are shown in Table 5. Once a sequence mutation was detected, a PCR assay was designed to detect the variant nucleotide. The PCR-based mutant assay allowed for the confirmation of sequence data and the determination of mutant allele frequency in a larger population sampling. Extended sampling for the identified mutation was restricted to lines containing *pvs-orf239* in high copy number.

The accession G-11115 from Mexico has a single transversion from C to A, resulting in a change in amino acid number 228 from leucine to isoleucine (mutation 1). In accessions G-23434A and G-7229, a mutation from G to T causes a change in amino acid number 161 from glutamate to aspartate (mutation 2). The G-23434A- and G-7229-associated mutation was found to be present in three additional accession lines based on PCR assay results. These accessions are G-23470 from Mexico and G-23434B and G-23441 from Guatemala. PCR amplification results from these mutants, as well as some of the nonmutant accessions, are shown in Figure 2. Three of the four wild accessions containing the identified mutation are grouped within the same genetic cluster based on the amplified fragment length polymorphism (AFLP) fingerprint results of TOHME *et al.* (1996). However, other lines within the same cluster, based on the AFLP data, do not show the same mutation. Likewise, the fourth mutated accession groups in a distinct AFLP cluster, although the separation was at only 15% dissimilarity. From these results one can speculate either that the mutations represent two independent events or that the mitochondrial DNA sequence data further refine the resolution of relationships within this portion of the AFLP-based phenogram. The latter explanation is likely the more reasonable on the basis of the low rates of point mutation observed within mitochondrial genomes (WOLFE *et al.* 1987). A similar PCR-based assay was performed to identify putative accessions with the mutation carried in the G-11115 accession. No other screened accessions were found to carry the same mutation.

To determine whether or not the detected *pvs-orf239* sequence functions as a sterility-inducing sequence, genetic crosses were made using the sterility maintainer (nonrestorer *frfr/fr2fr2*) line Sprite (BASSETT and SHUH 1982) as pollen parent. Accessions containing the sequence in high copy number were male fertile, presumably due to the presence of a fertility restorer nuclear genotype (this yet undefined restorer was designated *Frj*). Segregation ratios derived by testcrossing selected lines, some with high-copy-number *pvs-orf239* and some with low, are presented in Table 6.

From the results it was evident that the *pvs-orf239* sequence, when present in high copy number, functioned as a sterility-inducing sequence in both wild and cultivated accessions. The two identified *pvs-orf239* point mutations had no obvious effect on the function of the sequence in inducing sterility, and F_2 populations from crosses of two of the mutant lines (G-11115 and G-7229) with Sprite segregated for sterility. However, the presence of the sequence in low copy number did not induce sterility under the conditions of our study.

The *pvs-orf239* sequence may have originated by recombination substoichiometrically: Data from our sur-

TABLE 5

Accessions from which the *pvs-orf239* fragment was cloned and sequenced

CIAT accession no.	Country of origin	Status	Relative copy no. of *pvs-orf239*	Conservation relative to CMS-Sprite
G-11115	Mexico	Wild	High	Mutation 1
G-23434A	Guatemala	Wild	High	Mutation 2
G-24711	Colombia	Cultivated	High	100%
G-7229	Colombia	Cultivated	High	Mutation 2
G-6306	Ecuador	Cultivated	High	100%
G-13627	Mexico	Cultivated	Low	100%
G-12830	Ecuador	Cultivated	Low	100%
S-5257[a]	Peru	Wild	Low	100%

[a] This accession belongs to a group of *P. lunatus*-related species named *augusti.*

vey suggest that *pvs-orf239* represents a conserved sequence of ancient origin. To trace the evolution of the *pvs-orf239* sequence, we examined Phaseolus species more distantly related to *P. vulgaris* than those sampled by Hervieu *et al.* (1993). In *P. lunatus*, sequence analysis of *pvs-orf239* showed 100% sequence conservation with that of the CMS-Sprite line (data not shown). However, in *P. glabelus*, much more distantly related to *P. vulgaris* (Schmit *et al.* 1993), a possible progenitor form of the *pvs-orf239* sequence was identified. *P. glabelus* (accession

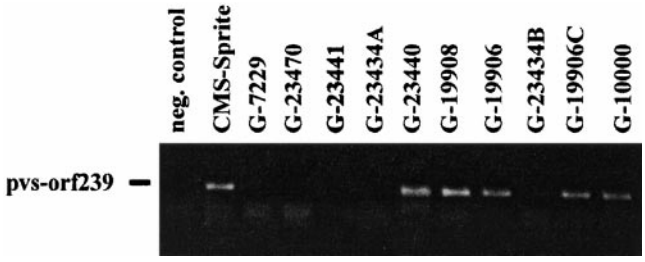

Figure 2.—PCR-based detection of a point mutation in accessions of *P. vulgaris* that contain *pvs-orf239* in high copy number. A PCR amplification assay was developed for the *pvs-orf239* sequence from mutants identified by sequencing. The assay exploits particular nucleotide substitutions that were identified using the primers *orf239-3′* with *cmsorf-5′*, shown here, and primers *orf239-5′* with *mutg-3′* (not shown). Primer sequences are listed in Table 3. Primers *cmsorf-5′* and *orf239-3′* amplify a 237-bp fragment from CMS-Sprite, and *cmsorf-5′* was designed to allow discrimination between CMS-Sprite and the mutant at the final 3′ nucleotide. Note that the sequence is amplified only from accessions in which the *pvs-orf239* sequence does not bear the mutation that was identified in accessions G-23434A and G-7229 by sequence analysis. Primers *orf239-5′* and *mutg-3′* amplify a 690-bp fragment from a mutant *pvs-orf239* sequence identified in line G1115 (reaction not shown here). The *mutg-3′* primer was designed with a nucleotide substitution at the 3′ end. PCR (MJ Research, Watertown, MA; PTC100) cycling conditions were an initial denaturation step of 95° for 5 min (hot start) followed by 25 cycles of 92° for 30 sec, 62° for 1 min, and 72° for 1 min and 30 sec, and a final extension step of 72° for 7 min. Reaction mixtures (50 μl total volume) consisted of 10 mm Tris-HCl, pH 9.0, 50 mm KCl, 2.25 mm $MgCl_2$, nucleotides dATP, dCTP, dGTP, and dTTP (125 μm each), 400 nm primers, 20 ng of template DNA and 1.25 units of *Taq* DNA polymerase (Promega).

S29161) contained a substoichiometric form of *pvs-orf239* that is incomplete, seemingly truncated at its 3′ end. Although the 5′ portion of the *pvs-orf239*-related sequence demonstrates 100% DNA sequence conservation to *pvs-orf239* from CMS-Sprite, PCR amplification of the 3′ end (terminal 140 bp) was consistently unsuccessful (data not shown). These results imply that the 3′ portion of the *pvs-orf239*-related sequence is either absent or highly diverged (Figure 3). Because the apparently truncated version of *pvs-orf239* from this accession of *P. glabelus* exists substoichiometrically, it was not feasible to directly clone mitochondrial fragments encompassing the region for more extensive analysis of its organization. We presume that the truncation point identified within *P. glabelus* represents a recombination site that may have participated in the evolution of *pvs-orf239* (Figure 3), and we are attempting to identify other distantly related lines with a similarly variant *pvs-orf239* 3′ sequence.

A DNA gel blot hybridization survey of other plant species for *pvs-orf239*-homologous sequences (not shown), together with database sequence similarity searches (Blast 2.1), allowed us to identify related nuclear and mitochondrial fragments in several plant species. A summary of these results is presented in Figure 3. A large stretch of homology with *Nicotiana tabacum* mitochondrial DNA implies that the majority of the *pvs-orf239* sequence at its 5′ end originated from mitochondrial sequences. An identified nuclear fragment from *P. vulgaris* with 100% homology to a small portion of *pvs-orf239* appears to represent a mitochondrial sequence originally derived from the region 5′ to the gene for the α-subunit of ATPase. In *P. vulgaris* this sequence has been transferred to the nucleus. The sequence identity of this domain was deduced by comparison to the soybean sequence, still residing within the mitochondrion. Of particular interest, many identified *pvs-orf239* sequence homologies terminated within a similar region ~270 bp internal to *pvs-orf239* as denoted in Figure 3. This site may represent a second recombination point involved in the evolution of the *pvs-orf239* sequence.

From these observations, we postulate that *pvs-orf239*

TABLE 6

Segregation for fertility restorer genes in *P. vulgaris* accessions

Genetic cross (female listed first)	F_2 segregation fertile:semisterile:sterile	χ^2 1:2:1[a]	*P*
G-11115/Sprite	10:20:5	1.97	0.35
CMS-Sprite/G-11115	12:26:6	3.08	0.20
CMS-Sprite//[CMS-Sprite/G11115]BC_1	5:3	0.5	0.49
G-24711/Sprite	6:11:4	0.48	0.55
CMS-Sprite/G-24711	5:11:9	1.76	0.40
CMS-Sprite//[CMS-Sprite/G-24711]BC_1	8:11	0.47	0.51
G-7229/Sprite	5:11:4	0.45	0.58
CMS-Sprite/G-7229	3:10:4	0.63	0.50
G-6306/Sprite	5:12:3	1.05	0.43
CMS-Sprite/G-6306	4:15:2	4.22	0.15
G-2525/Sprite	100% fertile		
CMS-Sprite/G-2525	7:15:4	1.31	0.41
CMS-Sprite//[CMS-Sprite/G-2525]BC_1	6:4	0.4	0.60
G-2518/Sprite	100% fertile		
CMS-Sprite/G-2518	3:10:4	0.64	0.50
G-12830/Sprite	100% fertile		
CMS-Sprite/G-12830[b]	2:12:3	2.99	0.22

[a] For BC_1 the χ^2 value was calculated for a 1:1 ratio.
[b] The segregation from this cross was based solely on pollen stainability data.

was formed from at least one major recombination event. Consequently, we suggest that *pvs-orf239* most likely originated from a truncated substoichiometric form that, following one to two recombination events to produce the present-day *pvs-orf239* form, was subsequently amplified to high copy number to become expressive.

The genomic shifting pattern is influenced by genome configuration: Physical maps of the mitochondrial genome of the sterility-inducing line CMS-Sprite and the revertant line WPR-3 were previously constructed using overlapping cosmid clones (Janska and Mackenzie 1993). To assess the degree of variation in genomic regions surrounding *pvs-orf239* in wild and cultivated populations of *P. vulgaris*, comparative DNA gel blot hybridizations were conducted with *pvs-orf239* and seven cosmid clones used as probes. The selected cosmid clones encompassed the recombination repeats and the regions flanking the *pvs-orf239* domain (Janska and Mackenzie 1993). Hybridization experiments included 13 of the lines shown to contain *pvs-orf239* in high copy number, and blots were prepared from total genomic DNA digested with *Pst*I and *Eco*RI enzymes.

The *pvs-orf239* sequence overlaps 6- and 4-kb *Pst*I restriction fragments in the CMS-Sprite line (Janska and Mackenzie 1993). The region immediately surrounding *pvs-orf239* appeared to be highly conserved in all lines. Most lines displayed the identical pattern

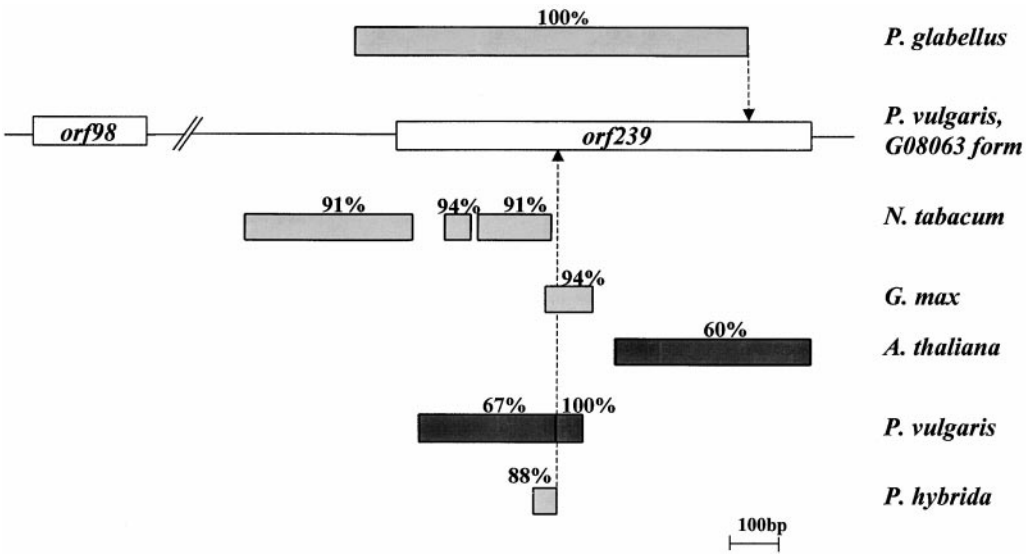

Figure 3.—Schematic comparison of *pvs-orf239* and its homologs detected in other plant species. Percentage of nucleotide identity is shown above the boxes and nuclear (solid box) and mitochondrial (shaded box) forms are indicated. Dashed lines designate two regions of predicted recombination suggested to give rise to *pvs-orf239*.

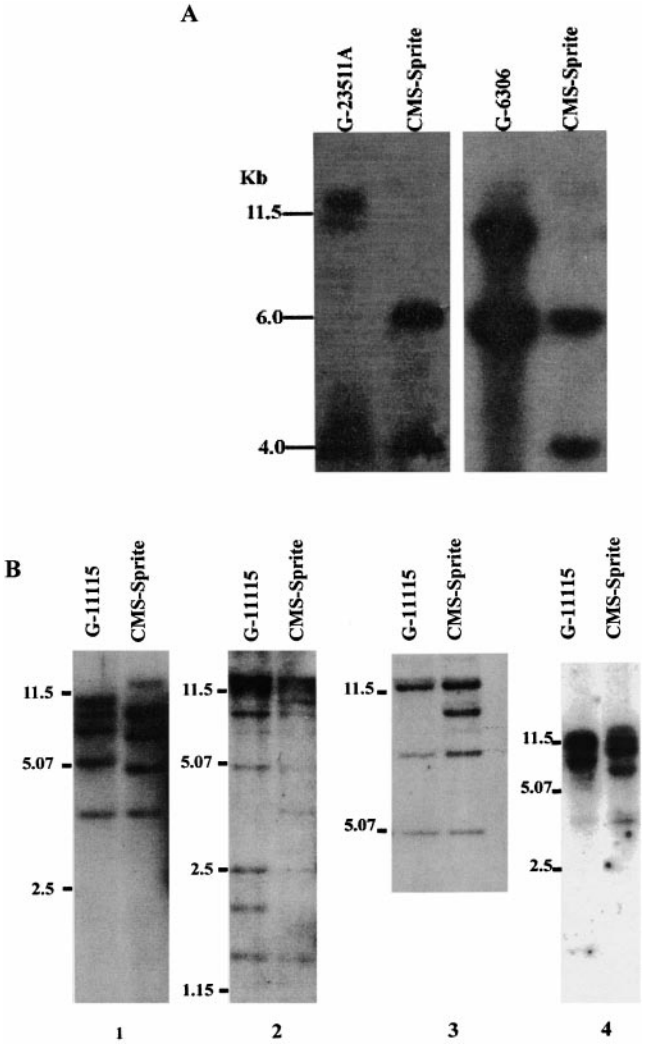

FIGURE 4.—Autoradiographs from total genomic DNA gel blot hybridization experiments. (A) Shown are two variant patterns observed in bean lines when hybridized with the *pvs-orf239* sequence as probe. The *pvs-orf239* sequence in CMS-Sprite spans adjacent 6.0- and 4.0-kb mitochondrial *PstI* fragments (JANSKA and MACKENZIE 1993). (B) An example of the different genomic environments found within the mitochondrial genome of bean accessions with high copy number *pvs-orf239* is shown. Line G11115 represents the type 2 environment in contrast to the type 1 environment found in CMS-Sprite. Panels 1–4 are the hybridization patterns derived by probing *Pst*I-digested genomic DNA with four different cosmids.

to CMS-Sprite, and two additional patterns arose as the apparent consequence of mutation within restriction sites. Accession G-6306 has lost a restriction site for the 4-kb *Pst*I fragment, whereas G-23511A has lost a site for the 6-kb *Pst*I fragment (see Figure 4).

Comparative hybridization experiments with cosmid clones revealed two distinct mitochondrial genomic configurations within the 13 accessions tested. It should be mentioned that some samples were probed with only a subset of the cosmids, so it is possible that additional variation went undetected in this study. Most of the accessions showed similar hybridization patterns to CMS-Sprite and these were classified as type 1. The type 2 group were polymorphic for four out of seven cosmid clones. An example of the two genomic forms is presented in Figure 4, with a summary of the experimental findings presented in Table 7.

TABLE 7

Two different mitochondrial genomic environments displayed by selected accessions containing *pvs-orf239* in high copy number

CIAT accession no.	Country of origin	Status	Pattern of mitochondrial genomic environment
G-11115	Mexico	Wild	2
G23470	Mexico	Wild	1[a]
G-19026	Mexico	Weedy	1
G-19026C	Mexico	Weedy	1
G-23511A	Mexico	Wild	1 and 2[b]
G-23434A	Guatemala	Weedy	2
G-23441	Guatemala	Wild	1
G-24929	Colombia	Weedy	2
OT-113	Colombia	Wild	1
G-24733	Colombia	Cultivated	2
G-24711	Colombia	Cultivated	2
G-7229	Colombia	Cultivated	1
G-6306	Ecuador	Cultivated	1

[a] Shared hybridization pattern with CMS-Sprite.
[b] Accession G-23511A shared hybridization pattern with CMS-Sprite for one cosmid used but differed in pattern for all others.

The two identified cytoplasm types did not appear to correlate with the origin of the accessions. Lines from the same gene pool contained distinct mitochondrial genomic patterns. One Mexican line that clustered within the Andean gene pool according to TOHME *et al.* (1996) showed both genomic environments. The line G-23511A contained the *pvs-orf239* region in a configuration identical to CMS-Sprite (type 1), but type 2 polymorphisms were detected for all other genomic regions compared. Likewise, accessions G-23441 and G-23434A, which displayed the same *pvs-orf239* point mutation, contained the *pvs-orf239* sequence within two different genomic configurations. On the basis of these data, we suggest that the origin of *pvs-orf239* in Phaseolus predates the mitochondrial genomic rearrangements that were identified in this study, and the observed polymorphisms may also be susceptible to stoichiometric shifting processes.

To test the influence of mitochondrial genomic configuration on the fertility restoration and copy number shifting process, the *Fr* nuclear fertility restorer gene was introduced by pollinations to two bean accession lines, G24711 and G11115. These lines were found to contain *pvs-orf239* within the type 2 genomic configuration distinct from CMS-Sprite. The experiment was de-

TABLE 8

Segregation for fertility restoration effected by the *Fr* gene in different *P. vulgaris* accessions

Genetic cross	F_2 segregation fertile:semisterile:sterile	χ^2 1:2:1	*P* value
[G-11115/Sprite]BC_1[a]//R-351[b]	5:7:4	0.325	0.7
[G-24711/Sprite]BC_1//R-351	3:8:4	0.190	0.96
CMS-Sprite/R-351	4:6:3	0.230	0.9

[a] Only sterile plants of BC_1 were crossed.
[b] R-351 refers to the [CMS-Sprite/R-351] BC_3F_3 plants used as source of *Fr* gene.

signed to investigate whether *Fr*-directed stoichiometric shifting of the *pvs-orf239* sequence and fertility restoration could occur in a line that contains high copy number *pvs-orf239* in a different genomic configuration. It should be noted, however, that the variant *pvs-orf239* genomic region detected within selected lines G-11115 and G-24711 was defined strictly by restriction fragment polymorphism analysis, with no physical mapping data available.

To carry out the experiment, male-sterile segregants were derived from these lines by crossing to a Sprite (*frfr*) pollen donor. These male-sterile plants were then crossed to the fertility restorer line R-351 (*FrFr*). The crossing strategy followed is presented in Table 8.

Phenotypic data from the crossing experiments were as predicted. Segregation data, presented in Table 8, show that introduction of nuclear gene *Fr* restored fertility to lines G-11115 and G-24711 and behaved as a single gene with incomplete dominance, consistent with what is observed in CMS-Sprite fertility restoration. The PCR assay for detection of the *pvs-orf239* sequence in high *vs.* low copy number was then performed with F_2 and F_3 fertile *Fr*-restored plants to determine whether introduction of the *Fr* gene influenced copy number of the *pvs-orf239* sequence. No copy number suppression of *pvs-orf239* was detected in either F_2 or F_3 fertile plants derived from crosses with accessions G-11115 or G-24711 as cytoplasm donors (Figure 5). However, copy number shifting of *pvs-orf239* was clearly evident within fertile F_2 plants derived from control crosses with CMS-Sprite as cytoplasm donor (Figure 5A). These results suggest that the genomic environment surrounding the *pvs-orf239* sterility-inducing sequence is important to the process of mitochondrial stoichiometric shifting and that the activities of genomic shifting and fertility restoration are functionally distinct.

Genomic shifting results in a 1000- to 2000-fold change in relative copy number: Mitochondrial genomic shifting is widespread throughout the plant kingdom, but the magnitude of the copy number changes is not well documented. To assess the difference in *pvs-orf239* copy number in CMS-Sprite *vs.* the spontaneous revertant line WPR-3, we utilized quantitative PCR in real time. Our approach was to compare the relative copy number of *pvs-orf239* in CMS-Sprite to that in WPR-3, to compare the copy number of *pvs-orf239* in each line to that of apocytochrome b (*cob*), and to compare *pvs-orf239* copy number in each line to that of the single copy nuclear gene for α-amylase. Total genomic DNA was prepared from etiolated seedlings and from young green leaves to assess the stability of relative copy number ratios over two developmental stages. To evaluate reproducibility, the identical experiment was conducted four times with CMS-Sprite and WPR-3, twice with α-amylase, and once with a second revertant line, 83-1.

Results of copy number estimations with quantitative PCR in real time are presented in Figure 6. Although we observed some differences between tissues, they were not striking. Overall, we observed a 1000- to 2000-fold reduction in *pvs-orf239* copy number upon reversion by comparing CMS-Sprite to WPR-3. Relative to a single copy nuclear gene, represented by α-amylase, *pvs-orf239* and *cob* in CMS-Sprite are both present in 30- to 200-fold excess. Because we have pooled cells for the DNA preparations, and we assume that mitochondrial numbers are not uniform in different cell types, these numbers are taken as averages across the various tissue types that were pooled. The observations from these experiments are consistent with previous estimates of mitochondrial genome copy numbers for a legume species presented by Lamppa and Bendich (1984). This previous study reported from 80 (seedling) to 260 (mature leaf) mitochondrial genome copies per cell in the pea plant.

Genomic shifting of *pvs-orf239* upon spontaneous reversion to fertility reduced *pvs-orf239* copy number to ~1/200 the copy number of α-amylase. These data imply that in lines containing *pvs-orf239* substoichiometrically, the sequence may be present in <1 per every 100 cells of young seedling or leaf tissues, since α-amylase should be present in only two copies per nondividing cell.

Evidence for higher levels of *pvs-orf239* within root meristem cells: Our data suggest that copy number-suppressed sequences within the mitochondrial genome may exist at far fewer than one copy per cell. However, the high incidence of *pvs-orf239* at substoichiometric levels within natural bean populations suggests that the sequence is efficiently transmitted and cannot be irreversibly lost from these lines. Therefore, plants

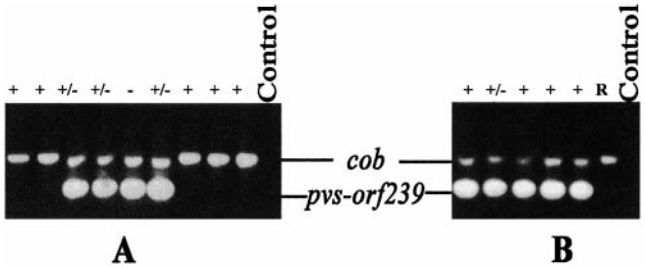

FIGURE 5.—PCR amplification of the *pvs-orf239* sequence in selected F_2 and F_3 plants derived from crosses to introduce the *Fr* restorer. (A) Shown are F_2 plants derived from the cross with CMS-Sprite as cytoplasm donor. (B) F_3 plants derived from crosses with G-11115 as a cytoplasm donor are shown. + designates fertile *FrFr* restored plants, +/− designates *Frfr* semisterile plants, − designates male-sterile plants, and R designates a spontaneous revertant included as a control sample. The control lane shows results of a reaction that included no template. Approximately 60 ng genomic DNA was used as template per reaction.

clearly must possess a means of ensuring the transmission of a complete mitochondrial genome to subsequent generations. The logical tissues in which this function would reside are the meristematic tissues that give rise to the ovule. An obvious and testable prediction of this model is the presence of *pvs-orf239* at high copy number within the apical meristem tissues of a line in which *pvs-orf239* is copy number suppressed in vegetative tissues.

To test this hypothesis, we isolated meristem tissues from WPR-3, a spontaneous revertant line in which *pvs-orf239* is substoichiometric, and CMS-Sprite, the isonuclear line from which WPR-3 was derived and in which *pvs-orf239* is present in high copy number. The isolation of large numbers of apical meristems precisely dissected from surrounding tissues was not feasible in *P. vulgaris*, so we were forced to conduct our study utilizing root meristems. Cytological studies by others comparing mitochondrial DNA replication in root and apical meristems of Arabidopsis have indicated striking similarity in mitochondrial behavior between these two meristem forms (FUJIE *et al.* 1993, 1994). Two meristem segments were isolated, representing the first (section 1) and second (section 2) 250-μm sections of the root tip (the root cap was not removed completely). These two segments meet at approximately the quiescent center (Figure 7A). DNA preparations from these two samples were assayed for *pvs-orf239* copy number, relative to *cob* and *coxI* (not

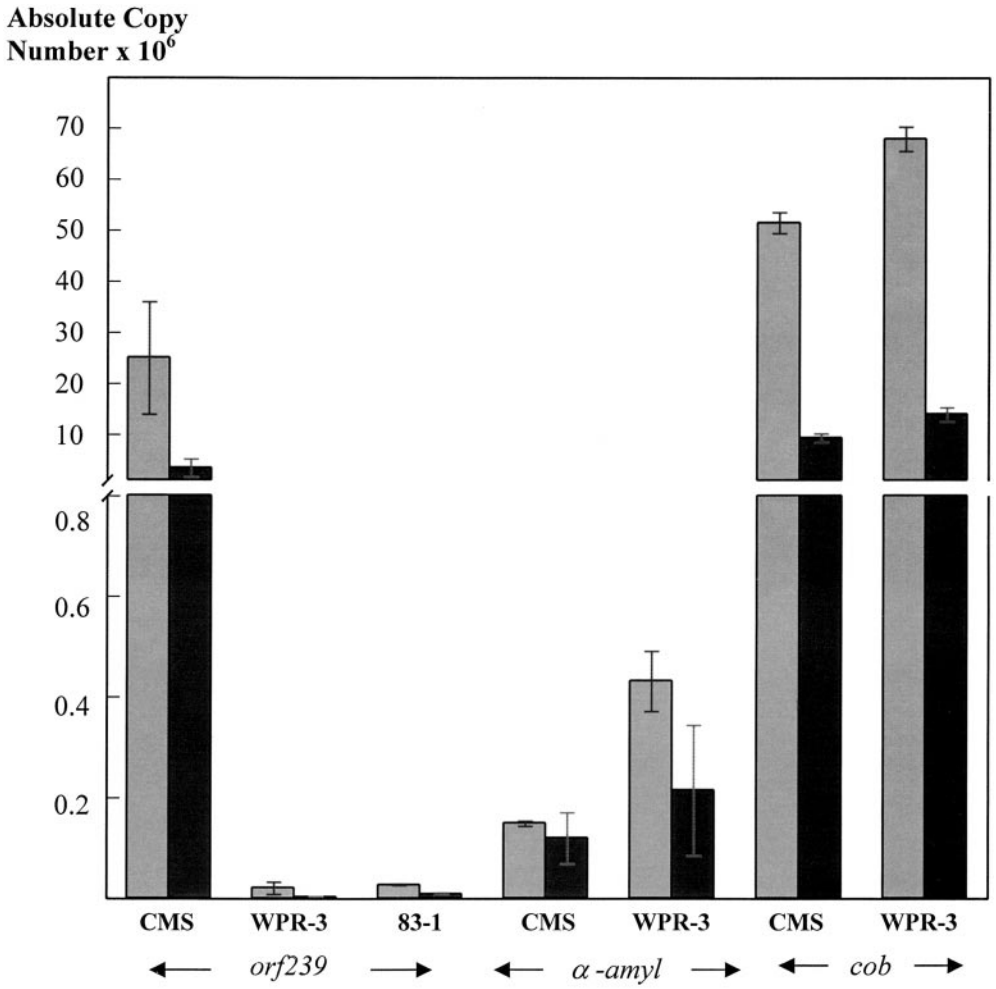

FIGURE 6.—Bar graph presentation of data obtained from quantitative PCR experiments in real time to estimate copy number of *pvs-orf239* relative to the mitochondrial apocytochrome b (*cob*) gene and the single copy nuclear α-amylase gene. Materials used in the study are the male-sterile line CMS-Sprite (*pvs-orf239* in high copy number) and two independent spontaneous revertant lines (*pvs-orf239* substoichiometric) WPR-3 and 83-1. Error bars represent experimental variation observed in two (α-amylase) or four independent experiments, with the exception of the 83-1 experiment conducted only once. Copy number estimates were conducted using total genomic DNA prepared from young seedling (solid box) and mature leaf (shaded box) tissues.

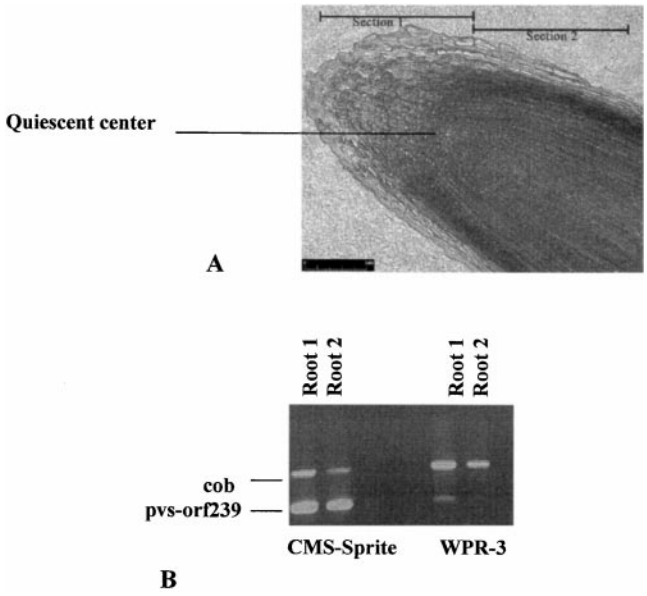

FIGURE 7.—(A) Longitudinal section of the root tip of common bean (×100). The approximate size and location of sections 1 and 2 for this study are indicated. (B) Agarose gel electrophoresis of PCR-amplified *pvs-orf239* and *cob* sequences from tissue of two sections (1, 2) of the root tip of CMS-Sprite and WPR-3 lines.

shown) as internal controls, by quantitative PCR in real time. It was not possible to precisely dissect the meristematic cells without root border cells, epidermal cells, and other differentiated cell types. Consequently, we regard each sample as merely enriched for undifferentiated meristem cells.

An example of results from our analysis is presented in Table 9. Not surprisingly, PCR amplification of the *pvs-orf239* sequence in samples from leaf tissues and section 2 root samples of the WPR-3 revertant line produced little or no detectable PCR product when viewed by gel electrophoresis. However, the *pvs-orf239* PCR product was readily visible in amplifications from WPR-3 section 1 root meristem samples (data not shown). This observation supports our hypothesis that particular cells within the meristem retain *pvs-orf239* in higher copy number. We assume that this "mitochondrial transmission zone" resides within the first 250 μm of the root meristem (section 1). In CMS-Sprite, the relative copy number of *pvs-orf239* in root sections did not differ significantly from that detected in leaf tissues (see Figure 6).

Quantitative analysis of *pvs-orf239* copy number within revertant WPR-3 *vs.* CMS-Sprite root meristem tissues indicated that the sequence is present in higher copy number within section 1 of the revertant root sample than in section 2 (Table 9). Yet, the *pvs-orf239* copy number in WPR-3 section 1 was still much lower than in CMS-Sprite section 1. We assume that this difference is attributable to the differentiated cells surrounding the meristem within both samples. This assay is not

TABLE 9

Estimated copy number of *pvs-orf239* and *cob* sequences in two sections of the root meristematic zone of WPR-3 line, based on quantitative real time PCR experiments

Root section	*Pvs-orf239*	*cob*
Section 1	2,870[a]	139,000
Section 2	155	240,000
Copy number ratio section 1 *vs.* section 2	19:1	0.58:1

[a] Each value represents the mean of two replicates in a single experiment.

sufficiently sensitive, due to imprecise meristem dissections, to determine whether *pvs-orf239* copy number in WPR-3 meristem cells is comparable to that in CMS-Sprite. We are currently attempting to better refine our resolution of the mitochondrial transmission zone in both root and apical meristems using *in situ* hybridization methods.

DISCUSSION

Cytoplasmic male sterility, as an agronomic trait of value to the hybrid seed industry, has been investigated genetically in a wide array of plant species. However, these studies have, with few exceptions, been centered on domesticated, highly selected plant materials. The opportunities to investigate the prevalence, evolution, and conservation of a sterility-inducing sequence in undomesticated populations have been relatively few. Because the molecular determinant of male sterility has not been defined for many of the systems in which natural populations can be assayed, investigators are limited to assessing cytoplasmic male sterility phenotype frequencies without the benefit of definitive genotype data. Moreover, those studies assessing the relative compensation in female fitness of the male sterility trait generally cannot take into account the influence of phenotype reversibility afforded by mitochondrial genomic shifting. From our observations it is reasonable to postulate that the equilibrium in frequency of male-sterile plants in a population would be largely influenced by environmental stimuli, perhaps temperature and insect pollinator frequency, that direct the genomic shifting process.

We have to date been unable to identify any other DNA sequences on the *pvs-orf239*-containing molecule that are unique to this molecule (JANSKA and MACKENZIE 1993; H. JANSKA and S. MACKENZIE, unpublished data). The extent to which *pvs-orf239* is conserved and distributed in the wild suggests that this sterility-associated sequence has likely provided some degree of selective advantage to these populations. Given that *P. vulgaris* is predominantly self-pollinated and that it originated within the mountainous regions extending from South-

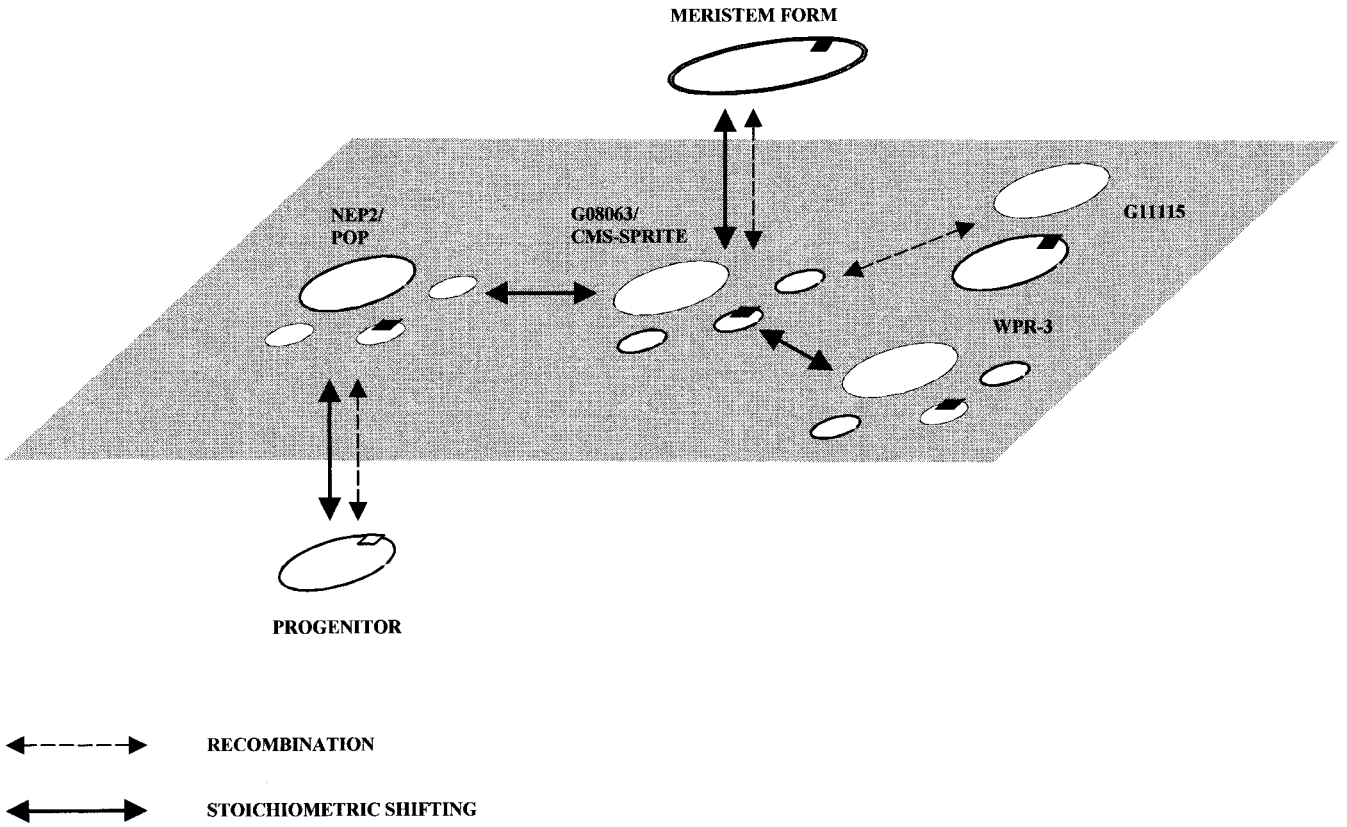

Figure 8.—Proposed model for mitochondrial genome evolution involving recombination and stoichiometric shifting in Phaseolus. A putative progenitor mitochondrial DNA configuration in Phaseolus predates the rearrangements that gave rise to the *pvs-orf239* sequence. We present the progenitor as a single, yet-unmapped molecule containing a progenitor form of *pvs-orf239* (open box). The existence of such a progenitor form is suggested by the data presented for *P. glabelus*. The progenitor form is predicted to give rise, via recombination and stoichiometric shifting, to the genome configuration identified to be present in the parents, NEP-2 and POP, of the sterility-inducing line G-08063. The form shown for NEP-2 and POP is based on physical mapping data presented in Janska *et al.* (1998). The mitochondrial genome configuration of line G-08063, based on physical mapping (Janska and Mackenzie 1993), is predicted to arise by stoichiometric shifting. Upon spontaneous reversion to fertility, a stoichiometric shifting event gives rise to the mitochondrial configuration identified in the fertile line WPR-3. The WPR-3 configuration is based on physical mapping data (Janska and Mackenzie 1993). The genomic shifting from G08063 and CMS-Sprite to WPR-3 occurs not only upon spontaneous reversion to fertility, but by the introduction of nuclear restorer gene *Fr* (Mackenzie and Chase 1990; Janska and Mackenzie 1993). An alternative, yet undefined, mitochondrial genome configuration is predicted in line G-11115. Data presented indicate that G11115 contains a different genomic configuration surrounding *pvs-orf239*. This genomic form is hypothesized to preclude spontaneous reversion or *Fr*-directed stoichiometric shifting of *pvs-orf239*. Finally, a distinct mitochondrial genome configuration is predicted to exist in the meristematic tissues of common bean, serving to ensure fidelity of mitochondrial genome transmission to subsequent generations. Dashed circles designate substoichiometric molecules; bold circles designate molecular forms in high copy number. The open box designates the *pvs-orf239* progenitor form; the solid box designates present-day *pvs-orf239*.

ern Mexico to Northern Argentina, one assumes that seed dispersal, largely by animals, often results in isolated populations. Taking this into account, we propose a model to address the observations made in this study.

In Figure 8 we outline a model for the possible role of mitochondrial stoichiometric shifting in the integration of male sterility to undomesticated Phaseolus populations. We postulate that the *pvs-orf239* sterility-associated sequence arose substoichiometrically, largely by recombination events. Putative progenitor forms of the sequence apparently remain within some distantly related species such as *P. glabelus*. This hypothesis is supported by similar observations in Brassicaceae, where mitochondrial recombination events are postulated to occur within low copy number molecules for subsequent amplification (Bellaoui *et al.* 1998).

At relatively low frequency, we have observed spontaneous genomic shifting of *pvs-orf239* to high copy number. This was previously hypothesized for the cross between lines POP and NEP-2, both containing *pvs-orf239* substoichiometrically, to give rise to G08063, in which *pvs-orf239* is amplified (Janska *et al.* 1998). We postulate that such sporadic amplification events continue to occur in natural populations, accounting for the frequency and broad distribution of high copy number *pvs-orf239* documented in our survey. However, once amplified,

pvs-orf239 appears to be managed by three strategies in nature. We assume that a large proportion of these lines contain the *Fr2* fertility restorer gene that was first identified in G08063 (Mackenzie 1991). This assumption is based on recent genetic analyses and DNA marker-based mapping studies that involved seven different accessions (M. Arrieta-Montiel and S. Mackenzie, unpublished data) as well as previously reported genetic analyses (Jia *et al.* 1997). However, we have also documented the incidence of spontaneous reversion to fertility, associated with the genomic shifting of *pvs-orf239* to substoichiometric levels (Mackenzie *et al.* 1988; Janska *et al.* 1998). Therefore, copy number suppression of *pvs-orf239* represents an alternate option for modulating expression of the sterility sequence. A final alternative that must be considered in light of results presented here is that *pvs-orf239* may become fixed in a high copy number state within particular populations. This is implied from our identification of lines in which *pvs-orf239* no longer demonstrates copy number suppression in response to the *Fr* locus.

These observations, taken together, suggest a scenario in which the *pvs-orf239* sequence, once amplified to high copy number, is generally suppressed in expression by the presence of nuclear fertility restorer genes such as *Fr2*. This restorer appears to suppress expression of the *pvs-orf239* sequence post-transcriptionally (Abad *et al.* 1995; Sarria *et al.* 1998). Infrequent incidents of outcrossing to a maintainer (nonrestoring) genotype, or the spontaneous amplification of *pvs-orf239* within a line containing a maintainer genotype, will result in a low frequency incidence of male sterility within a population. This would facilitate localized recombination activity. Should a male-sterile plant arise in isolation, the option for spontaneous reversion, producing a single seed-bearing pod on an otherwise sterile plant, would allow for propagation. However, in populations where the male sterility trait has produced a selective advantage, the sequence may now become fixed at high copy number by virtue of subsequent mitochondrial rearrangement events that locate *pvs-orf239* to a nondispensable molecule.

Superimposed on this model for the integration of the male sterility trait to populations is the ability to unerringly retain and transmit the sequence to the subsequent generation. Although we have not yet characterized the genome configuration of mitochondria within the postulated mitochondrial transmission zone of the meristem, we predict that the transmitted genome contains the complete genetic complement on a single replicative unit. This is not unlike the predicted "master" chromosome suggested to exist by others several years ago (Lonsdale *et al.* 1988) but never empirically demonstrated in later studies of mitochondria prepared from vegetative tissues or cell cultures (Oldenburg and Bendich 1996). Palmer and Shields (1984) first postulated that a simplified genomic structure would likely represent the transmitted form.

Although we present only speculation at this point, several predictions of our model can be tested. For example, a master transmitting molecule would be expected to differ markedly from the genome configuration in vegetative tissues with regard to the recombinationally active repeats assumed to give rise to the multipartite genome structure. Such repeats (in *P. vulgaris* there are two, Janska and Mackenzie 1993) would not be expected to be active in the predicted transmitting form of the genome. Moreover, all molecules found to be at substoichiometric levels within the Phaseolus genome, including that deriving from the progenitor NEP-2 and POP-type genomes (Janska *et al.* 1998), would presumably be present in equimolar amounts to the *pvs-orf239* region in the replicative genome form. We are testing these predictions presently.

Although additional studies will be required to more thoroughly test the validity of the proposed model, it is reasonable to assume that the introduction and integration of a mitochondrially encoded male sterility trait into a natural population would involve a multi-stage process. Our data and that of others' suggest that this process capitalizes on the mitochondrial DNA recombination and stoichiometric shifting activities that represent distinguishing features of the plant kingdom. Moreover, we suggest that much of this unusual genome activity is likely limited to vegetative stages of plant development, with the mitochondrial genome transmission process restricted to, and protected within, the meristem.

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LITERATURE CITED

Abad, A. R., B. J. Mehrtens and S. A. Mackenzie, 1995 Specific expression in reproductive tissues and fate of a mitochondrial sterility associated protein in cytoplasmic male-sterile bean. Plant Cell **7:** 271–285.

Bassett, M. J., and D. M. Shuh, 1982 Cytoplasmic male sterility in common bean. J. Am. Soc. Hortic. Sci. **107:** 791–793.

Bellaoui, M., A. Martin-Canadell, G. Pelletier and F. Budar, 1998 Low-copy-number molecules are produced by recombination, actively maintained and can be amplified in the mitochondrial genome of Brassicaceae: relationships to reversion of the male sterile phenotype in some cybrids. Mol. Gen. Genet. **257:** 177–185.

Bonhomme, S., F. Budar, D. Lancelin, I. Small, M. C. Defrance *et al.*, 1992 Sequence and transcript analysis of the *Nco* 2.5 Ogura-specific fragment are correlated with cytoplasmic male sterility in *Brassica* cybrids. Mol. Gen. Genet. **235:** 340–348.

Chase, C. D., and V. M. Ortega, 1992 Organization of ATPA coding and 3′ flanking sequences associated with cytoplasmic male sterility in *Phaseolus vulgaris* L. Curr. Genet. **22:** 147–153.

Fauron, C. M. R., M. Havlik and M. Casper, 1991 Organization and evolution of the maize mitochondrial genome. NATO ASI Ser. Ser. A Life Sci. **212:** 345–363.

Fujie, M., H. Kuroiwa, S. Kawano and T. Kuroiwa, 1993 Studies on the behavior of organelles and their nucleoids in the root apical meristem of *Arabidopsis thaliana* L. col. Planta **189:** 443–452.

Fujie, M., H. Kuroiwa, S. Kawano, S. Mutoh and T. Kuroiwa, 1994 Behavior of organelles and their nucleoids in the shoot apical meristem during leaf development in *Arabidopsis thaliana* L. Planta **194:** 395–405.

Gutierres, S., C. Lelandais, R. De Paepe, F. Vedel and P. Chetrit, 1997 A mitochondrial sub-stoichiometric *orf87-nad3-nad1* exonA co-transcription unit present in Solanaceae was amplified in the genus *Nicotiana*. Curr. Genet. **31:** 55–62.

He, S., A. Lyznik and S. Mackenzie, 1995 Pollen fertility restoration by nuclear gene *Fr* in CMS bean: nuclear-directed alteration of a mitochondrial population. Genetics **139:** 955–962.

Hervieu, F., L. Charbonnier, H. Bannerot and G. Pelletier, 1993 The cytoplasmic male sterility (CMS) determinant of common bean is widespread in *Phaseolus vulgaris* L. and *Phaseolus coccineus* L. Curr. Genet. **24:** 49–155.

Janska, H., and S. Mackenzie, 1993 Unusual mitochondrial genome organization in cytoplasmic male sterile common bean and the nature of cytoplasmic reversion to fertility. Genetics **135:** 869–879.

Janska, H., R. Sarria, M. Woloszynska, M. Arrieta-Montiel and S. A. Mackenzie, 1998 Stoichiometric shifts in the common bean mitochondrial genome leading to male sterility and spontaneous reversion to fertility. Plant Cell **10:** 1163–1180.

Jia, M. H., S. He, W. Vanhouten and S. Mackenzie, 1997 Nuclear fertility restorer genes map to the same linkage group in cytoplasmic male-sterile bean. Theor. Appl. Genet. **95:** 205–210.

Johns, C., M. Lu, A. Lyznik and S. Mackenzie, 1992 A mitochondrial DNA sequence is associated with abnormal pollen development in cytoplasmic male sterile bean plants. Plant Cell **4:** 435–449.

Kanazawa, A., N. Tsutsumi and A. Hirai, 1994 Reversible changes in the composition of the population of mtDNAs during dedifferentiation and regeneration in tobacco. Genetics **138:** 865–870.

Lamppa, G. K., and A. J. Bendich, 1984 Changes in mitochondrial DNA levels during development of pea (*Pisum sativum* L.). Planta **162:** 463–468.

Laser, B., S. Mohr, W. Odenback, G. Oettler and U. Kuck, 1997 Parental and novel copies of the mitochondrial *orf25* gene in the hybrid crop plant triticale—predominant transcriptional expression of the maternal gene copy. Curr. Genet. **32:** 337–347.

Lonsdale, D., T. Brears, T. Hodge, S. Melville and W. Rottmann, 1988 The plant mitochondrial genome: homologous recombination as a mechanism for generating heterogeneity. Philos. Trans. R. Soc. Lond. B **319:** 149–163.

Mackenzie, S. A., 1991 Identification of a sterility-inducing cytoplasm in a fertile accession line of *Phaseolus vulgaris* L. Genetics **127:** 411–416.

Mackenzie, S. A., and M. Bassett, 1987 Genetics of restoration in cytoplasmic male sterile *Phaseolus vulgaris* L. Theor. Appl. Genet. **74:** 642–645.

Mackenzie, S. A., and C. Chase, 1990 Fertility restoration is associated with loss of a portion of the mitochondrial genome in cytoplasmic male sterile common bean. Plant Cell **2:** 905–912.

Mackenzie, S. A., D. Pring, M. Bassett and C. Chase, 1988 Mitochondrial DNA rearrangement associated with fertility restoration and reversion to fertility in cytoplasmic male sterile *Phaseolus vulgaris* L. Proc. Natl. Acad. Sci. USA **85:** 2714–2717.

Oldenburg, D. J., and A. J. Bendich, 1996 Size and structure of replicating mitochondrial DNA in cultured tobacco cells. Plant Cell **8:** 447–461.

Palmer, J. D., and C. R. Shields, 1984 Tripartite structure of the Brassica campestris mitochondrial genome. Nature **307:** 437–440.

Pla, M., C. Mathieu, R. De Paepe, P. Chetrit and F. Vedel, 1995 Deletion of the last two exons of the mitochondrial *nad7* gene results in lack of the NAD7 polypeptide in a *Nicotiana sylvestris* CMS mutant. Mol. Gen. Genet. **248:** 79–88.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sarria, R., A. Lyznik, C. E. Vallejos and S. A. Mackenzie, 1998 A cytoplasmic male sterility-associated mitochondrial peptide in common bean is post-translationally regulated. Plant Cell **10:** 1217–1228.

Schmit, V., P. du Jardin, J. P. Baudoin and D. G. Debouck, 1993 Use of chloroplast DNA polymorphisms for the phylogenetic study of seven *Phaseolus* taxa including *P. vulgaris* and *P. coccineus*. Theor. Appl. Genet. **87:** 506–516.

Small, I. D., P. G. Isaac and C. J. Leaver, 1987 Stoichiometric differences in DNA molecules containing the *atpA* gene suggest mechanisms for the generation of mitochondrial genome diversity in maize. EMBO J. **6:** 865–869.

Small, I. D., R. Suffolk and C. J. Leaver, 1989 Evolution of plant mitochondrial genomes via substoichiometric intermediates. Cell **58:** 69–76.

Suzuki, T., N. Sasaki, A. Sakai, S. Kawano and T. Kuroiwa, 1995 Localization of organelle DNA synthesis within the root apical meristem of rice. J. Exp. Bot. **46:** 19–25.

Suzuki, T., S. Kawano, A. Sakai, A. Hirai and T. Kuroiwa, 1996 Variability of mitochondrial subgenomic molecules in the meristematic cells of higher plants. Genes Genet. Syst. **71:** 329–333.

Tohme, J., D. O. Gonzalez, S. Beebe and M. C. Duque, 1996 AFLP analysis of gene pools of a wild bean core collection. Crop. Sci. **36:** 1375–1384.

Vallejos, C. E., N. Sakiyama and C. Chase, 1992 A molecular marker based linkage map of *Phaseolus vulgaris* L. Genetics **131:** 733–740.

Vitart, M. V., R. De Paepe, C. Mathieu, P. Chetrit and F. Vedel, 1992 Amplification of substoichiometric recombinant mitochondrial DNA sequences in a nuclear, male sterile mutant regenerated from protoplast culture in *Nicotiana sylvestris*. Mol. Gen. Genet. **233:** 193–200.

Wolfe, K. H., W. H. Li and P. M. Sharp, 1987 Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast and nuclear DNAs. Proc. Natl. Acad. Sci. USA **84:** 9054–9058.

Wolstenholme, D. R., and C. M. R. Fauron, 1995 Mitochondrial genome organization, pp. 1–59 in *Advances in Cellular and Molecular Biology of Plants: The Molecular Biology of Plant Mitochondria*, Vol. 3. Kluwer Academic Publishers, Boston.

Yesodi, V., S. Izhar, D. Gidoni, Y. Tabib and N. Firon, 1995 Involvement of two different *urf-s* related mitochondrial sequences in the molecular evolution of the CMS-specific *S-Pcf* locus in petunia. Mol. Gen. Genet. **248:** 540–546.

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